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EMBRYO CRYOGENICS

Executive Summary - 2.1-7

Reuben J. Mapletoft

Western College of Veterinary Medicine

University of Saskatchewan

Saskatoon, Saskatchewan S7N 0W0

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A 4-year study was designed to investigate various factors that would affect embryo transfer pregnancy rates with frozen-thawed bovine embryos. Cost, success rate and use are all interdependent. Therefore, the overall objective of the study was to improve pregnancy rates following transfer of frozen-thawed bovine embryos and in turn reduce the cost and increase the utilization of embryo transfer by Saskatchewan Breeders. In the first series of experiments, it was clearly shown that storage of embryos in culture medium in the refrigerator for up to 24 hr was possible with no loss in viability after freezing. This has been of immense value to the embryo transfer industry as it is not always possible to freeze immediately after collection in the field. Several experiments were conducted to optimize the freezing process and to make more efficient use of time. Collectively, these experiments reduced the freezing process by approximately 75 min, a substantial saving in time and cost.

A series of experiments were conducted to determine the optimum method of thawing embryos. Generally speaking, survival in straws was high over a range of water-bath temperatures between +20°C and +35°C or in room temperature air (around 22°C). Glycerol must be removed from thawed embryos in a very time consuming 6-step dilution technique (60 min) or by a single-step with sucrose (10 min). These studies revealed that embryo survival did not differ with concentrations of sucrose ranging from 0.5M to 1.0M or with the modified 3-step procedure from the timing-consuming traditional 6-step procedure. Concentrations of sucrose below 0.5M or above 1.0M did result in reduced embryo survival.

A report from Australia suggested that sucrose could be toxic to embryos at high environmental temperatures. In a series of experiments involving a large number of frozen-thawed embryos these findings with frozen-thawed embryos were not confirmed. It was concluded that sucrose and environmental temperature was a non-issue because sucrose is never exposed to fresh embryos. Frozen-thawed embryos have been exposed to glycerol and this seems to protect embryos from the effects of sucrose at high temperatures. Thus, it is recommended that concentrations of sucrose between 0.5M and 1.0M are safe regardless of environmental temperature.

Serum is necessary in freezing medium but opinions vary as to its source. Results indicate that embryo survival did not differ whether BSA, fetal calf serum, newborn calf serum or regular bovine serum was used. In addition, lamb serum (a Saskatchewan product) and a ready-made commercial freezing medium containing BSA were equally efficacious. Therefore, cheaper, more readily available sources of serum are acceptable for embryo freezing.

Serum in freezing medium as a potential source of infectious disease is of concern to importing countries. In collaboration with Dr. B. Bavister, University of Wisconsin, the large molecular substance, polyvinyl alcohol (PVA) was found to be equally efficacious and embryo survival rates were high suggesting that this may be a satisfactory alternative for embryo exports.

Some of the most exciting results generated during these studies were in fast-freezing. A series of experiments were designed to investigate this potential new technique of freezing embryos. Embryos were exposed to various combinations of glycerol and sucrose for varying periods of time and then held in liquid nitrogen vapor for 5 min prior to plunging into liquid nitrogen. Indeed, a highly successful method of freezing mouse embryos in less than 10 min was developed. Unfortunately, this approach was not directly applicable to cattle embryos. The difference between the two species is likely a difference in permeability of embryo cells to cryoprotectants and water. The tremendous potential that this approach has to offer the freezing of cattle embryos makes this an area that requires additional research activity. It maybe possible to freeze cattle embryos in a procedure as simple as that of freezing bull semen. This would not only reduce costs but should also move the technology from the lab to the farm.

In summary, several important findings have been made in these studies. They are:

1. Embryos can be successfully refrigerated for 24 hr prior to freezing.
2. Embryos can be thawed in straws at water-bath temperatures between +20°C and +35°C or at room-temperature in air with equally high survival rates. *old.*
3. Glycerol maybe removed from thawed embryos with a 6-step process or with sucrose at concentrations between 0.5M and 1.0M or with 0.3M sucrose in a 3-step dilution process. ✓
4. Temperature and sucrose did not interact to damage embryos previously frozen in glycerol. ✓
5. Several kinds of sera including lamb serum produced in Saskatchewan can be safely used in freezing medium. ✓
6. Polyvinyl alcohol could replace serum as a nonbiological macromolecule in freezing medium. ✓
7. Commercially prepared and sold freezing medium can be reliably used to freeze embryos. ✓
8. Fast-freezing embryos in a solution of glycerol and sucrose is feasible. This could reduce time of freezing time from more than 90 min to less than 20 min. ✓

An analysis of commercial embryo transfer data indicates that frozen embryo transfer rates are now 10-15% below that of fresh embryos. This is made up of approximately 5-8% obviously destroyed in the freeze/thaw process and another 5-10% dieing after transfer. Clearly, over the 4 years of this contract success rates with frozen embryo transfer have improved approximately 10% and at least 1 hr has been taken off the freezing process.

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ABSTRACT

A 4-year study was designed to investigate various factors that would affect embryo transfer pregnancy rates with frozen-thawed bovine embryos. The overall objective of the study was to improve pregnancy rates following transfer of frozen-thawed bovine embryos and in turn reduce the cost and increase the utilization of embryo transfer by Saskatchewan Breeders. Important findings in these studies include:

1. Embryos can be successfully refrigerated for 24 hr prior to freezing.
2. Embryos can be thawed in straws at water-bath temperatures between +20°C and +35°C or at room-temperature in air with equally high success rates.
3. Glycerol maybe removed from thawed embryos with a 6-step process or with sucrose at concentrations between 0.5M and 1.0M or with 0.3M sucrose in a 3-step dilution process.
4. Temperature and sucrose did not interact to damage embryos previously frozen in glycerol.
5. Several kinds of sera including lamb serum produced in Saskatchewan can be safely used in freezing medium.
6. Polyvinyl alcohol could replace serum as a nonbiological macromolecule in freezing medium.
7. Commercially prepared and sold freezing medium can be reliably used to freeze embryos.
8. Fast-freezing embryos in a solution of glycerol and sucrose is feasible. This could reduce time of freezing from more than 90 min to less than 20 min.

An analysis of commercial embryo transfer data indicates that frozen embryo transfer rates are now 10-15% below that of fresh embryos. This is made up of approximately 5-8% obviously destroyed in the freeze/thaw process and another 5-10% dieing after transfer. Clearly, over the 4 years of this contract success rates with frozen embryo transfer have improved approximately 10% and at least 1 hr has been taken off the freezing process.

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ABSTRACT: A 4-year study was designed to investigate various factors that would affect embryo transfer pregnancy rates with frozen thawed bovine embryos. During the course of these studies several important findings have contributed to the success of bovine embryo transfer with frozen embryos. Embryo survival rates after thawing have increased by more than 10% and overall pregnancy rates have increased by 5-10%. Today, pregnancy rates with frozen-thawed bovine embryos are around 10% lower than that with fresh embryos whereas the difference at the beginning of this study approximated 20%. Furthermore, during the same period, the freeze-thaw procedure has been reduced by at least 1 hour making it more efficient and affordable.

BACKGROUND: The transfer of bovine embryos from superovulated donor animals to surrogate mothers has become a reliable method to rapidly propagate valuable genetics. Until recently the embryo transfer procedure has been limited to the transfer of fresh embryos. A reliable technique to freeze embryos for long-term storage and transportation over long distances has many advantages. This allows continuous collection of donors with transfer of embryos in a concentrated period dictated by the required calving period in that particular management situation. In addition, the ability to freeze embryos reduces the necessity of maintenance of a large recipient herd. Finally, the cost of international transportation of animals would be greatly reduced by transporting only frozen embryos and the resulting offspring would have the advantage of being raised by a cow indigenous to the area. In addition, regulatory officials require that embryos be held in a frozen state while donor animals are demonstrated free of infectious disease before the embryos are allowed into an importing country. The need for improved methods of freezing embryos with more reliable results is apparent.

In 1952 the first attempts to freeze mammalian embryos was reported and it was in 1972 that the first successful attempts to freeze mouse ova were reported. This group used a wide range of cooling and thawing rates, in the presence of glycerol or DMSO as cryoprotective agents, and found that when mouse embryos were cooled at 2°C/min., survival was very poor. When the cooling rate approximated 0.3°C/minute and warming rate of 10-15°C/min, embryo survival was very high. This demonstration of an optimum cooling and thawing rate for mouse embryos was very important in the development of further technology. Similar techniques resulted in

the successful freezing and thawing of rabbit embryos.

Although two calves were born from frozen embryos, initial research with cattle yielded poor results. In 1977, a successful program for the freezing of sheep embryos was applied to bovine embryos resulting in the birth of numerous calves. Utilizing the technology developed for the mouse, researchers have been able to consistently achieve 50% survival rate of frozen bovine embryos. However, considering a pregnancy rate of 60% of embryos transferred, an overall pregnancy rate of 30% of embryos frozen is much less than satisfactory. The successful utilization of embryo freezing will require that results approximate that of fresh embryos.

The deep-freezing of biological cells involves a dehydration process. Ice-crystals form in the fluids surrounding the cells from pure water. Salts then concentrate in the remaining water forming a hypertonic or hyperosmotic solution and embryonic cells respond osmotically by losing water. As the temperature cools, the ice crystals enlarge, the remaining salt solution becomes more concentrated and the embryo cells respond by losing more water. The dehydration of cells during freezing is important to prevent the formation of damaging ice crystals within cells when embryos are emersed in liquid nitrogen. The opposite occurs during thawing. As ice crystals melt, the salt solution bathing the embryo becomes less concentrated and the embryo responds osmotically by taking in water (rehydration).

Cryoprotectants are also used in the freezing of biological cells in order to protect them from the effects of ice crystal formation. Glycerol is a permeating cryoprotectant because it enters embryo cells and sucrose is a nonpermeating cryoprotectant because it will not enter embryo cells. In either case, cryoprotectants cause osmotic effects on cells and so care must be taken when adding and especially when removing cryoprotectants from embryos.

Normally, embryos are frozen in straws in a 1.5M solution of glycerol. Embryos are cooled to -7°C at which time ice crystals are induced in the medium bathing the embryos by touching the straw with a super-cooled forceps. After a period of 10 min during which time the ice crystal grows the embryos are cooled slowly at a rate between 0.3 and $0.8^{\circ}\text{C}/\text{min}$ to allow dehydration of the embryo. When a temperature between -30°C and -35°C is reached the straws are plunged directly into liquid nitrogen at -196°C . Embryos are usually thawed by placing straws in a water-bath at a temperature between $+20^{\circ}\text{C}$ and $+35^{\circ}\text{C}$. Glycerol is removed prior to culture or transfer by either dilution in 6-steps over 60 min or in a single-step over 10 min with a solution of sucrose, a non-permeating cryoprotectant. At the time of the initiation of these studies the freezing process took approximately $2\frac{1}{2}$ - 3 hr and pregnancy rates for bovine embryos ranged around 60% of those transferred

fresh and around 40% of those frozen, thawed and then transferred. Several steps in the procedure required improvement and still others required refinement from the point of view of efficient use of time and cost.

OBJECTIVES:

1. Improve the utilization of embryo transfer by cattle breeders;
2. Improve pregnancy rates following transfer of frozen thawed bovine embryos;
3. Investigate the various factors affecting embryo survival after freezing;
4. Elucidation of basic physical, biochemical and physiological properties of the embryo, its membranes, cryoprotectants and their interactions;
5. Improve Canada's competitive advantage in the exportation of genetic material.

METHODS, RESULTS AND DISCUSSION

Experiment 1A (Field Data): We are indebted to the University of Saskatchewan Reproduction Research Trust, Trans Tech Genetics Ltd., Saskatoon, and Sundown Livestock Transplants Ltd., Didsbury, Alberta for sharing data for analysis. In collaboration with Dr. H. Townsend a means by which embryo transfer data can be analyzed on an ongoing basis has been established. The overall objective of this exercise is to identify through multiple regressions, the relative importance of the various factors contributing to success (pregnancy) with fresh and frozen bovine embryos. All of the records for the University of Saskatchewan Reproduction Research Trust have now been analyzed (years 1982-84) and Trans-Tech Genetics Ltd. records for the years 1985-1988 have been entered. It is intended that subsequent years data will also be entered over time so that an ongoing monitoring of the various factors is possible. In addition, others in the commercial embryo transfer industry have expressed interest so it is hoped that most, if not all of the industry can be put on a similar record keeping system so as to broaden the data base from which we will be drawing conclusions. For the time being data for the years 1982-84 will be reported. Frozen embryos data will provide a historical basis by which progress, due in part to the studies reported herein, can be measured.

Complete records existed for a total of 1017 embryos transferred (172 frozen embryos and 845 fresh embryos). The 3-year average for pregnancy rate overall was 53.1% with the rate for fresh embryos (55.6%) being significantly higher than that for frozen embryos (40.7%; $P < 0.001$). It was not possible to determine embryo discard rate for frozen-thawed embryos. There was a significant effect of embryo stage and quality ($P < 0.001$) with the good and excellent quality embryos providing the highest pregnancy rates (58 and 45% respectively) compared to fair (41.5%) and poor (18.2%) quality embryos. Surprisingly, the body condition score

of recipients did not have a significant effect but fatter recipients did tend to have a reduced pregnancy rate. There was no difference whether the transfer was to the left or right horn of the recipient but the ease of transfer was significant with good or fair transfer results being significantly higher than poor transfers ($P < 0.01$). Recipient age was not significant but lactating recipients had a significantly higher pregnancy rate than did dry recipients (55.3 vs 48.4%; $P < 0.05$). Synchrony of recipients had no effect on pregnancy rate which is contrary to most published data. However, the numbers of recipients at 24-48 hours asynchrony were relatively small and this, no doubt, was at least partly responsible. Surgical transfers resulted in a pregnancy rate of 55.1% whereas nonsurgical transfers resulted in a pregnancy rate of 49.4% which only approached significance ($P = 0.09$) supporting our decision to do all nonsurgical transfers in 1984. Within the use of nonsurgical embryo transfers, the distance the embryo was placed down the uterine horn did not influence pregnancy rate but the length of time spent in the uterus did with the greater the time resulting in a lower pregnancy rate ($P < 0.001$). A subjective evaluation of the transfer was only marginally useful in predicting outcome ($P = 0.3$). Neither the length of time from collection to transfer nor the length of time from thawing to transfer affected results. However, we have learned that time was important and so all times were relatively short. Similarly, we have shown that refrigeration is a suitable alternative if embryos cannot be immediately transferred.

This is a modest beginning but does represent a great deal of work, the results of which is most gratifying. As additional data accumulate (beyond the terms of this contract), results will become more meaningful. We intend to report these data at the 1990 meeting of the International Embryo Transfer Society.

Experiment 1B (Field Data): Data shared by Sundown Livestock Transplants Ltd. indicate that frozen embryo transfers resulted in pregnancy rates 10-15% lower than fresh embryos. Surgical transfers were done and 1.5M glycerol was removed from frozen thawed embryos with 0.3M sucrose on a 3-step procedure. A total of 1290 embryos were frozen and 1183 were considered transferrable (a loss of 8%) resulting in 683 pregnancies (57% pregnancy rate). At the same time, the surgical transfer of 423 fresh embryos results in 283 pregnancies (67% pregnancy rate).

Experiment 2 (Field Data): To determine the effects (pregnancy rates) of glycerol dilution vs sucrose treatment for the removal of glycerol from frozen/thawed bovine embryos. We have previously shown that with both mouse and cow embryos survival in culture did not differ whether glycerol was removed by dilution (5-steps) or with sucrose in a single step. Dr. Martin Wenkoff, Altagenetics, Calgary kindly agreed to test these techniques on frozen/thawed bovine embryos in the field. Pregnancy rate of 163 bovine embryos in which glycerol was removed in a single step with 1.0M sucrose

for 10 min (53%) was not different from that of 63 frozen/thawed bovine embryos in which glycerol was removed in a five-step dilution process (57%). Field results support laboratory results and we now recommend that practitioners use the less time-consuming single step.

Experiment 3: To determine survival of bovine embryos cooled to 4°C prior to deep freezing. This experiment was originally supported up to \$16,000 by the Canadian Assoc. of Animal Breeders and the Alberta Agricultural Research Trust Fund. However, embryo quality and culture results made it necessary to replicate this experiment several times.

It is generally accepted that bovine embryos must be frozen within 6 hours of collection or survival rates will be very low. However, collections in the field or at other sites for export often make this time limit extremely difficult to meet. Recently, it has been shown that cooling both mouse and cow embryos to refrigerator temperature resulted in high survival rates as much as 48 hours later. Based on these data, we conducted an experiment with mouse embryos in which survival after freezing fresh was shown not to be different from that of embryos refrigerated 24 hours prior to freezing. The present experiment was designed to incorporate short term storage at 4°C into a freezing program for bovine embryos.

A total of 247 day 7 bovine embryos were used in this experiment. On each collection date embryos were grouped and sorted at random to be placed in one of four (4) experimental groups. Group I embryos were placed directly into Ham's F-10 culture medium and evaluated after 24 hours. Group II embryos were frozen directly after collection and upon thawing were cultured as in Group I. Group III embryos were placed in Dulbeccos PBS culture medium, refrigerated for 24 hours and then cultured for 24 hours as in Group I. Embryos in Group IV were refrigerated for 24 hours as in Group III, frozen as in Group II, thawed and cultured for 24 hours as in Group I.

Results were somewhat equivocal and follow in table form:

	Group I Culture	Group II Freeze & Culture	Group III Refrigerate & Culture	Group IV Refrigerate, Freeze & Culture
No. embryos	52	83	58	54
No. surviving refrig. (%) -	-	-	58 (88)	51 (94)
No. surviving freezing (%) -	-	80 (96)	-	48 (89)
No. surviving 24 hr culture (%)	41 (79)	31 (37)	12 (21)	24 (44)

Of particular concern is the survival rate after 24 hours in culture of 37% for frozen/thawed embryos. Our results usually exceed 50%. Similarly, survival in culture of previously refrigerated embryos (Group III) of 21% is also very low. The survival of embryos that had been refrigerated, frozen, thawed and then cultured (Group IV) approached acceptability at 44%. We find these results very difficult to interpret. At the very least, refrigerated and then frozen embryos (Group IV) were not worse off than embryos frozen fresh (Group II).

Work in this area has continued because of the belief by others (Liebo, Texas) that 0°C (crushed ice) was a superior temperature to 4°C (refrigerator) to maintain embryos prior to freezing. In part I, mouse embryos were divided to be placed in the refrigerator (n = 238) or in ice water at 0°C (n = 241) for 24 hr. After this time embryos were further divided to be either cultured or frozen to be later thawed and cultured. As can be seen from the following table there were no differences in embryo survival suggesting that the refrigerator is suitable to maintain embryos prior to freezing.

	0°C		4°C	
	<u>Culture</u>	<u>Freeze/culture</u>	<u>Culture</u>	<u>Freeze/culture</u>
n	130	111	134	104
% survival				
24 hr	73	63	80	63
48 hr	64	63	85	54

In part II, 144 bovine embryos were frozen directly or maintained at 0°C or 4°C for 24 hrs prior to freezing. Survival was determined in culture over 48 hrs. Results were not different as can be seen from the following table.

	Groups		
	<u>Freeze</u>	<u>4°C/Freeze</u>	<u>0°C/Freeze</u>
n	63	50	31
% survival			
1 hr	57	24	42
24 hr	35	8	19
48 hr	5	2	7

Embryo quality was a problem and variability was broad. However, there seems to be no effect of cooling for 24 hr prior to freezing. This is now a standard, recognized procedure and it was developed at W.C.V.M.

Experiment 4: To develop the optimal method of thawing embryos in straws. The rationale for this experiment was that although most embryo-transfer practitioners are using a 35°C water-bath to thaw embryos in straws, this technique had been developed for embryos in glass ampules. Theoretically, embryos in straws should be thawed in a 20°C water-bath because of the faster rate of temperature conduction.

In this experiment 505 mouse embryos were frozen in either 0.25 ml semen straws, 0.5 ml semen straws or 1.0 ml glass ampules. Embryos were then thawed in a 20°C, 25°C, 30°C or 35°C water-bath with mild agitation for 40 sec. and glycerol was removed with 1.0M sucrose at room temperature for 10 min. Embryos were placed in culture for 48 hr. Results (survival in culture) were as follows:

Freezing Container	Thaw Temperature							
	20°C		25°C		30°C		35°C	
	24hr	48hr	24hr	48hr	24hr	48hr	24hr	48hr
0.25 ml straws (%)	35/53 (66)	30/53 (57)	26/48 (54)	22/48 (46)	19/50 (38)	21/50 (42)	16/36 (44)	15/36 (42)
0.50 ml straws (%)	21/52 (40)	23/52 (44)	21/37 (57)	15/37 (41)	24/51 (47)	28/51 (55)	17/47 (36)	19/47 (40)
1.0 ml glass Ampules (%)	24/55 (44)	22/55 (40)	16/45 (36)	15/45 (33)	2/15 (13)	1/15 (7)	1/16 (6)	3/16 (19)

Generally speaking, there were no differences demonstrated at any temperature. However, there was a tendency for reduced survival in culture at 35°C, especially when embryos were frozen in glass ampules.

A second experiment (4B) was designed to determine the optimum thawing temperature for 0.25 ml straws. A total of 432 mouse morula were frozen in a 1.5M glycerol solution in 0.25 ml. straws. Straws were thawed in a water-bath at 20°C, 25°C, 30°C or 35°C for 40 sec and glycerol was removed with a 1.0M sucrose solution prior to culture in BWB medium. The 20°C thaw resulted in a superior embryo survival rate after 48 hr in culture as seen in the following table.

n	Thaw Temperature			
	20°C	25°C	30°C	35°C
% survival	106	109	109	108
24 hr	89	72	85	80
48 hr	91	76	81	76

This result was predictable based on Schneider's permeability equations. A third experiment (4C) was designed to determine whether embryos required time after thawing to respond osmotically to sucrose. A total of 342 mouse morula were frozen in the usual manner. After thawing in a 20°C water-bath, embryos were placed in a 1.0M sucrose solution for 10 min immediately, 10 min or 20 min after thawing. There were no differences among groups as to embryo survival in culture (immediately - 80%; 10 min - 83%; 20 min - 80%). Time need not be a consideration when processing embryos after thawing. Membranes appear to be completely functional immediately after thawing.

To take the thawing information to its conclusion, a final experiment (4D) was designed to investigate thawing rates of a 1.5M glycerol solution in 0.25 ml. straws. We are grateful to Dr. U. Schneider, Hanover, Germany for his assistance in calculating thawing rates. The determinations were carried out by freezing 0.2 ml of a 1.5 M glycerol/PBS solution in a 0.25 ml straw by placing it into liquid nitrogen (-196°C). A 30g thermocouple was located in the middle of the straw. The straw was subsequently placed in a water-bath at the temperature indicated and the water-bath was stirred or left undisturbed. A temperature recorder was set at a speed of 200 mm/min. The thawing curves were all nearly superimposable over a temperature range between -196°C and -20°C. Differences exist mainly between -20°C and the water-bath temperature. Stirring effects were negligible between -196°C and -20°C but thawing rates were always higher when stirring was used. The numerical values of the calculated thawing rate differed widely and depended on the temperature range used for the calculation. The results are consistent with expectations. Thawing rates under stirred conditions are always higher than under unstirred conditions. In a 0°C ice-water bath and unstirred conditions, the thawing rate was markedly reduced due to the build up of ice outside the straw, forming an additional insulation layer. The following table shows these differences.

		Stirred				Unstirred			
Temp		-80°C	-60°C	-80°C	-60°C	-80°C	-60°C	-80°C	-60°C
Range		-10°C	-10°C	-2°C	-2°C	-10°C	-10°C	-2°C	-2°C
Water-Bath Temperature									
35°C	2000	1500	1231	940	1448	1111	835	644	
30°C	1909	1500	1017	790	1312	1000	742	580	
25°C	1615	1250	900	696	1312	1032	659	511	
20°C	1500	1363	780	610	840	681	454	348	
5°C	823	625	362	276	608	461	174	131	
0°C	626	476	191	145	617	652	99	74	

These data tend to confirm why we have had problems determining an optimum temperature to thaw 0.25 ml straws. Thawing rates do not in fact differ by much. It can be concluded from these data that reports of thawing rates in the literature are not terribly reliable. It is probably much better to report thawing conditions e.g. water-bath temperature and whether or not stirring was used and the length of exposure of the straw to the thawing conditions.

At the same time as these experiments were being initiated, a report appeared in the literature which suggested that there was an interaction of temperature and sucrose concentration in that higher concentrations of sucrose appeared to reduce embryo survival at higher temperatures i.e. around 35°C. Not only did this suggest that the 35°C water-bath could be killing embryos, but certain export situations to tropical countries could result in environmental temperatures in the 35°C range which could not help but affect embryo survival. It was felt imperative that we investigate these interactions.

Experiment 5: In the first experiment (5A) mouse embryos were frozen in 0.25 ml semen straws and thawed in a 20°C, 27°C or 35°C water-bath and glycerol was removed at that same temperature with 0.75M or 1.0M sucrose for 10 min. Embryos were then placed in culture and evaluated at 24 hr. Survival in culture was as follows:

Thaw and Sucrose Temperature

Sucrose Conc.	<u>20°C</u>	<u>27°C</u>	<u>35°C</u>
0.75M (%)	28/50=(56%)	26/48=(54%)	26/63=(41%)
1.0M (%)	28/50=(56%)	27/58=(47%)	35/66=(53%)

Data were inconclusive but did suggest that 20°C is superior to 35°C from the point of view of embryo survival in culture - not nearly as profound as was reported in the literature. An additional experiment (5B) was designed to investigate the two extremes i.e. 20°C and 35°C, and 1.0M sucrose. Again no significant differences were forthcoming. Survival rates after 24 hrs. in culture were as follows:

	Thaw and Sucrose Temp.	
	20°C	35°C
Survival	72/130	68/134
%	55.4%	50.7%

In this series of experiments we have seemed to be unable to demonstrate an interaction of temperature and sucrose concentration. However, the fact that this has been reported in the literature, and the potential extreme importance of this phenomenon suggests that this line of investigation should be continued, especially in the cow. Therefore, another experiment (5C) was designed to determine the effects of time on the proposed interaction of temperature and sucrose concentration on embryo survival in culture. Nine hundred and twenty-seven (927) mouse embryos were frozen as previously described and were allocated into one of 18 groups for thawing. Embryos were thawed at 20°C, 30°C or 35°C and placed in a 0.5M or 1.0M sucrose solution at the same temperature as thawing for 5, 10 or 30 minutes. Embryos were then washed and placed in culture. After 24 hours in culture embryo survival was determined by morphological appearance and development. Results are reported in the following table.

		Temperature								
		20°C			30°C			35°C		
		5	10	30	5	10	30	5	10	30
Sucrose	Time									
0.5M		53	59	66	73	70	70	60	75	71
(n)		(40)	(70)	(62)	(51)	(50)	(50)	(58)	(40)	(45)
1.0M		64	58	64	85	75	70	75	80	60
(n)		(58)	(60)	(45)	(52)	(53)	(54)	(56)	(46)	(37)

Statistically, there tended to be a reduced survival of embryos in 1.0M sucrose at 35°C for 30 minutes compared to 5 and 10 minutes. This tends to support our earlier findings in which 35°C also tended to yield inferior results. Of additional concern is an apparent and statistically significant decrease in embryo survival at 20°C as compared to 30°C and 35°C. Results are much more subtle than previously reported but are suggestive in every experiment. This series of experiments was reported in abstract form at the 24th Annual Meeting of the Society for Cryobiology in Edmonton.

Experiment (5D) was designed to confirm the originally reported results i.e. utilizing fresh (unfrozen) embryos. A total of 407 mouse embryos at the morula stage of development were exposed (fresh) to 0.5, 1.0 or 2.0M sucrose at either 20°C or 35°C for either 10 min or 30 min and then cultured in BWB culture for up to 48 hours. From the following table, one can see that 2.0M sucrose was toxic to embryos at 35°C and 20°C. When the exposure was 1.0M sucrose for 10 min. embryo survival and development was very good but 1.0M sucrose at 35°C tended to be toxic. Similarly, 1.0M sucrose at 20°C for 30 min or 0.5M sucrose at 35°C for 30 min tended to be toxic to embryos.

Temp. Time	Sucrose					
	0.5M		1.0M		2.0M	
	20°C	35°C	20°C	35°C	20°C	35°C
10 min	32/33 (97)	32/34 (94)	30/33 (90)	19/37 (51)	21/34 (62)	0/32 (0)
30 min	35/36 (97)	29/37 (78)	25/36 (69)	4/34 (12)	0/27 (0)	0/34 (0)

Data support the originally published report. In both cases, sucrose was exposed to fresh embryos and sucrose concentration, temperature and time all influenced embryo survival. The real question is why frozen/thawed embryos were more able to tolerate the effect of sucrose when we in fact felt they may be more susceptible to be effects of sucrose?

We feel that results may be related to the rate at which water leaves the embryo and the rate at which the embryo contracts. Water will move through membranes much more quickly than will glycerol. Therefore, sucrose can be expected to have a much more profound effect on embryos that had not been previously exposed to glycerol.

Although we are still not sure of the practical significance of these findings, we have revealed some very important basic cryobiological information. We intend to continue this work with fresh mouse embryos and frozen and fresh bovine embryos. At the very least it is a matter of degree i.e. the rate at which embryos contract when embryos are exposed to sucrose. Certainly the potential exists for embryo damage. We will investigate whether a slower rate of contraction is less damaging and whether bovine embryos are more susceptible to the damaging effects of sucrose than are mouse embryos.

Experiment 6: To compare two methods of removing glycerol from frozen/thawed mouse embryos with sucrose. We have previously shown that in both mouse and cow embryos, survival in culture did not differ whether glycerol was removed in a single step with sucrose or in five steps with glycerol dilutions. Others have now reported another method in which sucrose and glycerol dilution were combined in three steps. A total of 412 mouse embryos were frozen in 1.5M glycerol. Embryos were allocated at random to have glycerol removed after thawing in a single step with 1.0M sucrose for 10 min. or in three steps (0.75M glycerol and 1.0M sucrose for 5 min; 0.375M glycerol and 1.0M sucrose for 5 min.; 1.0M sucrose for 5 min). Embryos were then washed and placed in culture for 24 hours. Results in the following table demonstrate that there was no difference in embryo survival regardless of treatment. There is no support for the more time-consuming methods of removing glycerol.

	Glycerol Removal	
	<u>One-Step Sucrose</u>	<u>Three-step Sucrose</u>
Survival in culture	82/204	76/188
%	40%	40%

However, many within the industry are using the three-step approach and are claiming superior results. Still others are suggesting that a 0.5 M solution of sucrose may be superior to the 1.0 M solution of sucrose. Finally, we have shown that embryos may be thawed at a wide range of temperatures but highest survival rates were obtained when embryos were thawed in a 20°C water-bath. Again, industry people are claiming superior results with a room-temperature air thaw. A series of experiments have been designed to investigate these various possibilities.

In the first experiment (6B) 214 mouse embryos were frozen in the usual manner and thawed in a 25°C water-bath. Glycerol was removed with sucrose at one of four concentrations ranging from 0.25 M to 2.0 M. Embryos were placed in BWB culture medium for 48 hr. Results are shown in the following table.

Sucrose Conc. (M)	Number Embryo	# Embryo Surv. After 48 Hr in Culture	Survival Rate %
0,25	53	24	45,3 ^b
0,50	51	34	66,6 ^a
1,00	59	42	71,2 ^a
2,00	58	8	13,8 ^c

abc - $P < 0,05$

Sucrose concentrations at a level of 0.5 M or 1.0 M yielded the highest survival rates. Clearly, 0.25 M sucrose is too low and 2.0 M sucrose is too high in concentration. The former probable causes osmotic shock and the latter probably causes solution effects and may also cause "leaking" of membranes.

In a second experiment (6C) mouse embryos were frozen to investigate two (2) methods of thawing embryos in 0.25 ml straws (22°C water-bath or 22°C room-temperature air) and four (4) different methods of removing 1.5M glycerol after thawing (1.0M sucrose, 0.5M sucrose, 0.3M sucrose or 0.3M sucrose with decrease concentrations of glycerol in three-steps (as in previous experiment). Embryos were washed three times and placed in BWB culture medium to be evaluated for survival at 48 hr. Results appear in the following table.

Glycerol Removal	Method of Thawing	No. of Embryos	No. with* Broken zona	Survival Rate(%)
1.0M Sucrose	22°C water	57	4	82.4 ^a
	22°C air	54	2	81.5 ^a
0.5M Sucrose	22°C water	52	6	75.0 ^a
	22°C air	50	0	64.0 ^{ab}
0.3M Sucrose	22°C water	54	8	46.3 ^b
	22°C air	56	0	44.6 ^b
3-steps in 0.3M sucrose	22°C water	60	5	84.3 ^a
	22°C air	58	1	88.3 ^a

ab - Percentages with superscripts not in common are different

The 0.3M sucrose solution was obviously insufficiently hypertonic to remove 1.5M glycerol from embryos. There seemed to be no differences in other methods of glycerol removal or between the two methods of thawing or embryo survival.

A large experiment was initiated with cow embryos to investigate thawing methods and methods of glycerol removal. In the first experiment (6D) 121 embryos were frozen by a standard method and thawed at room temperature (22°C) or at 25°C in water-bath. Glycerol was removed with 1.0M sucrose for 10 min (group 1), 0.5M sucrose for 10 min (group 2), decreasing concentrations of glycerol and 0.3M sucrose in three-steps of 5 min each (group 3) or 0.3M sucrose for 10 min (group 4). Embryos were cultured for 24 hr in PBS and evaluated for survival as reported in the following table.

Group	1 1.0M Sucrose	2 0.5M Sucrose	3 0.3M Sucrose (3-step)	4 0.3M Sucrose (1-step)	Total
Thaw					
Air 22°C	11/15 73%	11/15 73%	11/12 92%	12/15 80%	45/57 79%
Water bath 25°C	9/14 64%	11/17 65%	10/16 63%	7/17 41%	37/64 58%
Total	20/29 69%	22/32 69%	21/28 75%	19/32 59%	82/121 68%

Although data were considered preliminary, it did seem that an air-thaw may have yielded higher embryo survival rates than a water-bath thaw, that 0.3M sucrose has too low a concentration and that the three-step approach may have yielded slightly higher survival rates. A second more definitive experiment (6E) was designed to confirm the above data. A total of 365 bovine embryos were frozen in the usual manner in a 1.5M glycerol solution in 0.25 ml straws. Embryos were thawed at 22°C in a water-bath for 1 min or in room air for 5 min. Glycerol was removed in one of four ways as described above and after washing of embryos they were placed in Ham's F-10 medium for culture. After 48 hr in culture embryos were evaluated for survival and development. Results are described in the following table.

Group	I 1.0M Suc.	II 0.5M Suc.	III 0.3 Suc. (1-step)	IV 0.3 Suc. (3-steps)	Total
Thaw at 22° C Air	28/46 61% ^a	35/50 70% ^a	14/39 36% ^b	34/53 64% ^a	111/188 59%
Water- bath	31/47 66% ^a	27/38 71% ^a	13/38 34% ^b	38/54 70% ^a	109/177 62%
Total	59/93 63% ^a	62/88 70% ^a	27/77 35% ^b	72/107 67% ^a	220/365 60%

ab - percentages with superscripts not in common are different (P<0.05)

Broken zonae - Air: 2.6%; Water: 22%

There seems to be little difference between the air thaw or water-bath thaw at 22°C or between 1.0M sucrose and 0.5M sucrose or 0.3M sucrose in a 3-step dilution. Clearly, the 0.3M sucrose solution in 1-step is not sufficiently hypertonic to remove 1.5M glycerol from bovine embryos.

It is tempting to suggest that 0.5M sucrose or 0.3M sucrose in a 3-step dilution are superior to 1.0M sucrose but differences were not different. There was no difference in method of thaw but the air thaw resulted in significantly fewer broken zona pellucida. This did not seem to affect embryo survival but could be an important consideration in preventing disease transmission with embryo transfer. The intact zona pellucida is necessary to keep disease organisms away from the embryo. Therefore, washing procedures, designed to remove potential pathogens, must be done prior to freezing.

Experiment 7: Several other lines of investigation were followed. Three experiments were designed to investigate the effect of protein and protein source on embryo survival in culture or during freezing. In the first experiment (7A) four sources of protein were compared as to their ability to support embryonic development in culture. Embryonic survival after 48 hours in culture for crystalline bovine serum albumin (BSA; 103/129; 79%), BSA Fraction V (52/60; 87%), 15% fetal calf serum (FCS; 91/129; 71%) and for BSA and FCS (85/97; 88%) did not differ. Results at 24 hr. in culture were even more tightly grouped. Results suggest that any one of these protein sources can be successfully used in the culture of embryos.

In a second experiment (7B) the above four protein sources were compared in a freezing medium containing Dulbecco's phosphate

buffered saline (PBS) and 1.5M glycerol and all ere compared to a commercial freezing medium consisting of PBS, BSA and 1.5M glycerol (Gibco's freezing medium). Embryos were frozen in the usual manner, thawed in 25°C water-bath and glycerol was removed with 1.0M sucrose. Embryos were placed in BWB Culture Medium for 48 hours. Embryo survival in culture did not differ suggesting that all these protein sources may be successfully used in an embryo freezing medium and that a commercial embryo freezing medium is equally efficacious. Results appear in the following table:

Survival (%)	Protein Source				
	FCS	BSAcryst	FCS&BSA	BSAV	Gibco
	69/82	69/88	68/97	80/106	81/105
	(84)	(78)	(70)	(76)	(77)

The latter observation i.e. the effect of the commercial medium, is of particular importance to the commercial embryo transfer industry.

Fetal calf serum is becoming expensive and so an Ontario company (Bocknek) and a Saskatchewan company (D & J Biologicals), Moose Jaw are looking at entering the market. The former has a bovine serum and the latter has a lamb serum. These sera were compared to commercial FCS and BSA in two experiments. In the first experiment (7C) these two products resulted in slightly depressed survival rates of mouse embryos in culture for 48 hours. We learned after this trial that the sera had not been heat-inactivated. After heat-inactivation embryo culture results were most acceptable, especially with lamb serum.

Survival (%)	Protein			
	BSA	Lamb Serum	Bovine Serum	FCS
	32/37	37/37	24/37	24/37
	(86)	(100)	(65)	(65)

Data look very promising for lamb serum (a Saskatchewan product). We intend to work further with this group in the establishment of this product on the market.

Experiment 8 was designed to determine if ice crystallization must go to completion before embryos are cooled after ice crystal nucleation. Several new cheap embryo freezers have become available. Their principle is simple in that cooling rate can be controlled by holding an alcohol bath a certain distance above the surface of liquid nitrogen. However, it is very difficult to hold the temperature at -7°C for any period of time so that ice nucleation (seeding) can go to completion. The question is whether the prescribed period of 10 min is necessary. A total of 458 mouse morula were placed in a 1.5M glycerol solution in 0.25ml straws to

be frozen in the usual manner. At seeding temperature (-7°C) ice nucleation was induced and cooling was resumed immediately or after 5, 10 or 15 min. Embryo survival in culture after thawing are shown in the following table.

	<u>Immediate</u>	<u>Time</u> <u>5 min</u>	<u>10 min</u>	<u>15 min</u>
n	169	87	98	104
% survival				
1 hr	40	68	76	83
24 hr	38	75	76	60
48 hr	40	77	79	62

Results suggest very strongly that a period of time is required for ice crystals to form after seeding. However, results also suggest that too long a period of time at -7°C (15 min) may also be detrimental. This could be a very important observation and we are replicating this experiment before we go on to pursue these studies further.

It is a well established fact that the slow freezing of embryos is a dehydration process and that we add cryoprotectants to the freezing medium to protect the embryos during this slow cooling, dehydration process. It is also well documented that sucrose is a nonpermeating cryoprotectant and that when embryos are added to a sucrose solution embryos dehydrate as water is drawn out in response to the osmotic differences between the inside and the outside of the embryo cells. It stands to reason then that sucrose could be used to dehydrate embryos prior to rapid freezing. Others have now shown that this is possible.

The first experiment (9A), was based on previously published procedures. A total of 380 mouse morula were exposed to a solution of 3.0M glycerol and 0.5M sucrose for 20 min and loaded into 0.25 ml straws. Straws were then plunged directly into liquid nitrogen or held in the neck of the liquid nitrogen tank at -30°C to -50°C for 5 min. prior to plunging into liquid nitrogen or laid horizontally on a steel plate in the liquid nitrogen vapor at -160° for 5 min prior to plunging into liquid nitrogen. Embryos were thawed in a 20°C water bath and glycerol was removed at room temperature in a 0.5M solution of sucrose for 12 min. Embryos were cultured in BWB medium for 48 hr. We were surprised to find that the direct plunging of embryos resulted in a survival rate of 45%. The other two methods resulted in an embryo survival rate of 53% each. These results were very promising and so we felt we should continue to pursue this line of investigation.

A second experiment (9B) was designed to determine the optimum freezing temperature for embryos in the neck of the liquid nitrogen container. A total of 238 mouse embryos were loaded in straws in 3.0M glycerol and 0.5M sucrose and held in the neck of the liquid nitrogen tank at three levels prior to plunging into liquid

nitrogen. After thawing and removal of glycerol with 0.5M sucrose embryos were evaluated after 48 hr in culture with BWB medium. Embryo survival rates ranged from 58 to 72% with the level approximating -60°C providing the best results. This level was selected for subsequent studies.

In the third experiment (9C) 179 mouse embryos were placed in 3.0M glycerol and 0.5M sucrose in straws and frozen either vertically at -60°C or horizontally at -54°C . After thawing glycerol was removed with 0.5M sucrose for 12 min and embryos were placed in BWB culture medium for 48 hr. Embryo survival for straws in vertical position (77%) was not different from that in horizontal position (86%). Both were considered to be very good.

In the fourth experiment (9D) a total of 2251 mouse morula were randomly placed into one of 24 treatment groups in order to determine the optimal glycerol and sucrose concentrations for fast freezing of mouse embryos and to determine the optimal concentration of sucrose to remove glycerol from embryos after thawing. Embryos were placed in the prescribed concentrations of glycerol and sucrose in 0.25 ml straws for 20 min prior to freezing. Straws were then held in the neck of the liquid nitrogen container at -54°C for 5 min. prior to plunging into liquid nitrogen. Straws were thawed in a 25°C water bath and glycerol was removed with sucrose at room temperature. Embryos were placed in BWB culture medium and evaluated at 48 hr. Results and the experimental design are outlined in the following table 8. Briefly, embryo survival improved as glycerol concentration increased and as sucrose concentration decreased. There was no significant difference between 3.0M and 3.5M glycerol or between 0.25 and 0.5M sucrose. Sucrose in the dilution medium at a concentration of 0.5M tended to yield higher survival rates than at a concentration of 0.75M.

Sucrose Conc (M)	Glycerol Conc. (M)											
	2.00			2.50			3.00			3.50		
	0.25	0.50	0.75	0.25	0.50	0.75	0.25	0.50	0.75	0.25	0.50	0.75
Dilution Sucrose												
0.50 M												
n	109	72	114	98	83	87	78	91	94	107	107	98
%	45 ^d	29 ^e	2 ^f	65 ^c	74 ^{bc}	30 ^e	85 ^{ab}	85 ^{ab}	28 ^e	90 ^a	82 ^{ab}	22 ^e
0.75 M												
n	95	90	84	104	84	82	92	94	98	83	109	98
%	43 ^d	21 ^e	2 ^f	68 ^c	73 ^b	18 ^e	83 ^{ab}	76 ^{ab}	15 ^e	82 ^{ab}	69 ^b	15 ^e

abcdef - means with superscripts not in common are different ($P < 0.05$).

A fifth experiment (9E) was designed to refine the glycerol concentrations and to determine whether horizontal freezing (as is done with semen) is just as efficacious. Mouse embryos (n = 396) were frozen in straws containing 2.75 to 3.50M glycerol and 0.4M sucrose. Straws were placed horizontally in the vapor of a liquid nitrogen tank at a temperature between -50°C and -60°C for 5 min and then plunged into liquid nitrogen. After thawing at 25°C embryos were placed in 0.5M sucrose for 12 min and then placed in BWB culture for 48 hr. The percentage of embryos developing to the blastocyst stage did not differ but 3.25M glycerol seemed to yield optimal results.

	Glycerol Conc (M) plus 0.4M Sucrose			
	<u>2.75</u>	<u>3.00</u>	<u>3.25</u>	<u>3.50</u>
n	95	94	93	94
% survival	88	89	87	92

These are the highest embryo survival rates tht we have ever achieved after freezing mouse embryos.

These results were very promising and the ease with which embryos could be frozen in this way has made it imperative that we pursue this approach with bovine embryos. Two experiments using bovine embryos were conducted. In the first (9F) 96 bovine embryos were frozen with one of three combinations of glycerol and sucrose horizontally at -54°C and then plunged into liquid nitrogen. Glycerol was removed with 0.5M sucrose and embryos were cultured for 24 hr in Ham's F-10 medium. The overall results were disappointing. However, the combination of 2.8M glycerol and 0.5M sucrose yielded the best survival (28%) with the combinations of 3.2M glycerol and 0.4M sucrose (6.5%) and 3.2M glycerol and 0.5M sucrose (10.7%) not different from one another.

In the second experiment (9G) an attempt was made to define glycerol concentration more closely. Embryos were frozen in 0.4M sucrose and 2.0M, 2.5M or 3.0M glycerol, horizontally at -54°C. Glycerol was removed with 0.5M sucrose and embryos were cultured in Ham's F10. Results, again disappointing, are shown in the following table.

	Glycerol Conc (M)		
	<u>2.0</u>	<u>2.5</u>	<u>3.0</u>
n	10	10	10
% survival			
1 hr	20	50	50
24 hr	10	0	20

It was difficult to understand why bovine embryos did not seem to survive this approach to freezing when mouse embryos did with a high rate. Bovine embryos are less permeable than mouse embryos

to glycerol. Therefore, based on the different permeability coefficients for bovine and mouse embryos, we elected to investigate various times for equilibration with the high concentrations of glycerol and sucrose before rapid freezing.

In a third experiment (9H) day-7 bovine embryos (morulae to blastocysts) rated as good and excellent quality were washed 10 times in PBS containing serum and antibiotics. Embryos were prehydrated by exposing them to a 1.5 M glycerol solution in PBS for 5 min and then transferred into a solution of 3.0 M glycerol and 0.5 M sucrose for 5 min (groups A and B), 10 min (group C) or 15 min (group D). During this period of time, embryos in each group were loaded into 0.25 ml straws. At the end of the equilibration time, group A straws were plunged directly into liquid nitrogen while groups B, C, and D straws were placed in liquid nitrogen vapors at -54°C . After 5 min straws were then plunged into liquid nitrogen.

Straws were thawed in a 20°C water-bath for 1 min and embryos were placed in a 0.5 M sucrose solution for 10 min. Embryos were washed four times in PBS and placed in Ham's F-10 culture medium at 37°C for 48 hr. Embryos which developed from morulae to blastocysts to expanding blastocysts were judged to be viable. In the first replicate of 12 bovine embryos, all approaches showed great promise as follows.

Freezing (%)	Treatment	Equilibration	Survival
<u>Method</u>	<u>Group</u>	<u>Time</u>	
One-step	A	5	2/3 (67)
Two-step	B	5	2/3 (67)
Two-step	C	10	2/3 (67)
Two-step	D	15	3/3 (100)

All groups had a very high survival rate in culture and so it seemed that the problem of fast-freezing bovine embryos was simply a question of gradual introduction of glycerol and equilibration time with the glycerol-sucrose freezing medium. We went ahead and froze several more replicates of bovine embryos in the same way.

TREATMENT	EQUILIBRATION TIME ^a	AFTER THAWING	1 hr	24 hr	48 hr
A (%)	5 min ^b	39/42 (92%)	16/43 (37%)	3/43 (6%)	1/43 (2%)
B (%)	5 min ^c	41/43 (95%)	23/43 (53%)	8/43 (13%)	5/43 (11%)
C (%)	10 min ^c	36/43 (83%)	20/43 (46%)	6/43 (13%)	4/43 (9%)
D (%)	15 min ^c	29/32 (90%)	12/32 (37%)	1/32 (3%)	0/32 (0%)

- (a) Embryos were exposed to 1.5 M glycerol for 5 min and then to 3.0 M glycerol and 0.5 M sucrose for the equilibration times.
- (b) Straws plunged directly into liquid nitrogen
- (c) Straws held in liquid nitrogen vapors for 5 min before plunging

From this table it is clear that survival of embryos in culture after 24 and 48 hr was extremely poor and so we have not yet arrived at the correct formula for fast-freezing bovine embryos. Why the different results in the first replicate is not clear. We did change technical staff because the student who was working on this project returned to China. There could be subtle changes in the technique which were not related to protocols. We have discussed this problem with Dr. Uli Schneider and he feels that in one or more areas we are likely on the edge of an embryo survival window and that a change in technical staff has caused us to topple to the embryo death side. We are now looking into the various components of the procedure in order to determine the weak link.

If one looks at embryo survival immediately after thawing, it is indeed very high. The embryos seemed to deteriorate very fast once they were placed in culture. The embryos responded osmotically as they would if membranes were intact during the removal of glycerol with sucrose. We generally feel that this is the first sign of survival after the freeze-thaw process. Does this mean that damage is occurring after thawing? We were able to delay the degeneration by delaying the time to when the embryos were placed in the incubator. The technique does still look promising and does potentially have a lot to offer but the problems are perplexing and will require a lot of painstaking work.

The relative failure to successfully fast-freeze bovine embryos led us to question whether the approach was a sound one or not. Therefore, we decided to freeze mouse embryos in exactly the

same way to determine if mouse embryo survival would be high. Results presented in the following table indicate that the approach is probably sound. Survival rates were not as high as in previous mouse experiments but this could be due to the change in technical staff.

TREATMENT	EQUILIBRATION TIME ^a	AFTER THAWING	1 hr	24 hr	48 hr
A (%)	5 min ^b	30/42 (71%)	18/43 (42%)	18/42 (42%)	18/42 (42%)
B (%)	5 min ^c	36/41 (85%)	25/42 (59%)	21/42 (50%)	21/42 (50%)
C (%)	10 min ^c	38/41 (90%)	16/41 (38%)	12/42 (28%)	11/41 (26%)
D (%)	15 min ^c	29/42 (69%)	19/42 (45%)	18/42 (42%)	15/42 (32%)

- (a) Embryos were equilibrated in 1.5 M glycerol for 5 min before placement in freezing medium of 3.0 M glycerol and 0.5 M sucrose
- (b) Straws placed directly into liquid nitrogen
- (c) Straws placed in liquid nitrogen vapors for 5 min before plunging

It should be noted that embryo survival after 48 hr in culture seemed to be highest in the group B i.e. equilibration for 5 min and then placement in liquid nitrogen vapours for 5 min before plunging into liquid nitrogen. Dr. Schneider felt that this would likely be the case as one would want to freeze embryos before equilibration was complete i.e. while dehydration due to the high concentrations of glycerol and sucrose was maximal. We then designed an experiment, with mouse embryos, in which a still lesser equilibration period was used. Mouse embryos were exposed to 1.5 M glycerol for 5 min and then to 3.0 M glycerol and 0.5 M sucrose for 1 min (groups A and B), 5 min (group C) or 10 min (group D). Groups C and D (5 and 10 min equilibration time) had similar survival rates to those in the previous experiment but the 1 min equilibration times seemed to result in improved survival rates as illustrated in the following table.

TREATMENT	EQUILIBRATION TIME ^a	AFTER THAWING	1 hr	24 hr	48 hr
A (%)	1 min ^b	29/43 (67%)	28/43 (65%)	28/43 (65%)	28/43 (65%)
B (%)	1 min ^c	35/46 (76%)	35/46 (76%)	35/46 (76%)	33/46 (71%)
C (%)	5 min ^c	20/47 (42%)	18/47 (34%)	16/47 (34%)	16/47 (34%)
D (%)	10 min ^c	28/45 (62%)	19/45 (42%)	17/45 (35%)	14/45 (31%)

- (a) Embryos were equilibrated in 1.5 M glycerol for 5 min before placement in freezing medium of 3.0 M glycerol and 0.5 M sucrose
- (b) Straws were placed directly into liquid nitrogen
- (c) Straws placed in liquid nitrogen vapours for 5 min before plunging

This supports the view that shorter equilibration times allowing for maximal dehydration of embryos is the approach that we should be taking. Once we have a better handle on this approach we will have to go back to bovine embryos. We will also have to do osmotic studies with photography in order to study the osmotic effects of various concentrations of cryoprotectants on bovine embryos.

This work showed a great deal of promise and did, in our minds, have a lot to offer the embryo transfer industry. It had the potential of reducing the embryo freezing procedure to a simplicity equivalent to that of freezing semen and would reduce the time required for freezing embryos from more than 1.5 hrs to less than 20 min. This would not only reduce costs but would also move the technology to the farm.

Because the fast-freezing bovine embryos was beyond the scope of this contract, it was decided that this line of research would be better funded on a specified project basis. A proposal for grant support was submitted to the Saskatchewan Agriculture Development Fund and it was not funded. Unfortunately, this promising line of investigation must be temporarily abandoned until grant support can be found. In the meantime, results to date are being written up for publication.

CONCLUSIONS

During the tenure of this contract a series of experiments have been conducted, all directed to an improvement in the success and utilization of frozen bovine embryo transfer. In summary, several important findings have been made in these studies. They are:

1. Embryos can be refrigerated for 24 hr prior to freezing.
2. Embryos can be thawed in straws in water-bath temperatures between +20°C and +35°C or at room-temperature in air.
3. Glycerol maybe removed from thawed embryos with a 6-step process or with sucrose at concentrations between 0.5M and 1.0M or with 0.3M sucrose in a 3-step dilution process.
4. Temperature and sucrose did not interact to damage embryos previously frozen in glycerol.
5. Several kinds of sera including lamb serum produced in Saskatchewan can be safely used in freezing medium.
6. Polyvinyl alcohol could replace serum as a nonbiological macromolecule in freezing medium.
7. Commercially prepared and sold freezing medium can be reliably used to freeze embryos.
8. Fast-freezing embryos in a solution of glycerol and sucrose is feasible. This could reduce time of freezing from more than 90 min to less than 20 min.

An analysis of data from commercial embryo transfer practitioners indicates that frozen embryo transfer rates are now 10-15% below that of fresh embryos. This is made up of approximately 5-8% obviously destroyed in the freeze/thaw process and another 5-10% dieing after transfer. Clearly, over the 4 years of this contract success rates with frozen embryo transfer have improved approximately 10% and at least 1 hr has been taken off the freezing process. To that extent we have achieved some of our objectives.

IMPACT

The impact that these studies have made on the embryo transfer industry and the cattle industry generally has been considerable. As alluded to above, more than 1 hr has been taken off the freezing of bovine embryos and success rates with frozen-thawed bovine embryos are now such that pregnancy rates are within 10% of that of fresh bovine embryos. This has resulted in reduced costs and increased use of bovine embryo transfer inspite of a depressed agricultural economy. Of particular note is the dramatic increase in the use of frozen embryos for the export of Canadian genetics. During the 4-year period of this contract embryo exports have increased from 605 in 1985 to more than 12,000 in 1988. The work done at the University of Saskatchewan and supported by the Saskatchewan Agriculture Development Fund has brought considerable

attention and prestige to the University and the province. Through these studies we have gained both national and international recognition and are looked upon as the source of embryo freezing information for the entire country.

PUBLICATIONS, PRESENTATIONS AND OTHER BENEFITS

During the tenure of this contract considerable research data, in the area of embryo cryobiology, have been generated. As cows must be superovulated to produce embryos for freezing, considerable data on superovulation have also been generated. A listing of articles (professional and lay) in the two areas is enclosed. The Saskatchewan Agriculture Development Fund is acknowledged wherever possible.

Three Chinese graduate students sponsored by CIDA (2) and the Government of Saskatchewan (1) have completed their theses on work supported under this contract. In addition, Dr. Mapletoft has initiated a post-graduate course entitled "Principles of Embryo Transfer 801.3". Dr. Mapletoft has participated in Embryo Transfer/Freezing courses in Chile, Argentina, Mexico, USA and Canada as well as special programs put on by the American Assoc. of Bovine Practitioners for North American Veterinarians and Western Breeders for Russian Veterinarians. In all cases work supported under this contract features strongly. Dr. Mapletoft has participated in Agriculture Canada Technical/Trade Missions to Russia, India and Indonesia and has represented Sask. Agdevco in Technical Seminars in Czechoslovakia and Mexico. In addition, he has briefed various delegations on embryo transfer-freezing technology for Sask. Agdevco, Sask. Agriculture, Sask. Economic Development and Trade and Saskatchewan Advanced Education and Manpower. Dr. Mapletoft has been active in the International Embryo Transfer Society (IETS) being past-chairman of its Import/Export Committee and led a delegation from the IETS to the OIE in Paris. He was founding president of the Canadian Embryo Transfer Assoc. and a past-member of its board of Directors. He actively participated in the Program Review of Agriculture Canada's Embryo/Infectious disease research and he has participated (once as committee chairman) on NSERC site visits of programs in embryo technology. He is regularly consulted by governments, the veterinary profession, industry, embryo transfer practitioners, breeders and producers from within Saskatchewan, Canada and continental North America. The work conducted under this contract has, in part, helped to establish this level of recognition and expertise. Although many of the direct benefits of these studies impact on others as much or more than Saskatchewan Agriculture, indirect benefits to Saskatchewan such as stature and recognition do exist. In addition, breeders, scientists and government people from around the world come to Saskatchewan because of our expertise. This can hopefully result in increased economic activity.

Theses

- K. Xu, M.Sc. 1988. Fast-freezing of Mouse Morula - supported by CIDA - Semex Canada.
- M. Wu, M.Sc. 1988. Superovulation in Beef Cattle - supported by CIDA - Semex Canada.
- H. Wang, M.Sc. 1988. Superovulation in the cow with PMSG - supported under Saskatchewan - Jilin Agreement.

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- R.J. Mapletoft, 1987. Embryo Transfer Management: Pharmacologic Management of Donors and Recipients, Bovine Reproduction. Proceedings of a Symposium 13th Annual Food Animal Medical Conference. The Ohio State University, Columbus.
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- K. Xu, A. Palasz, J. Moker and R.J. Moker, 1988. Fast Freezing of Mouse Morulae. Theriogenology, 29, 334 (Abstract)
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- R.J. Mapletoft, 1989. Embryo Transfer Technology. Canadian Dairy Technical Seminar. New Delhi, India and Jokarta, Indonesia (1 hr).
- R.J. Mapletoft, 1989. Superovulation in the cow. IETS Meeting, Chairman of Panel Discussion, San Diego.
- R.J. Mapletoft, 1988. Embryo transfer in the cow: Technology update. INTA Castellar, Argentina (8 hrs).
- R.J. Mapletoft, 1988. Superovulation in the cow and the freezing of bovine embryos. AABP Annual Meeting - Embryo Transfer Update, Calgary (3 hrs).
- R.J. Mapletoft, 1988. Embryo Transfer Technology. Canadian Dairy Technical Seminars, Moscow and Kiev, USSR (2 hr).
- R.J. Mapletoft, 1988. The Canadian Embryo Transfer Industry Engineers Society, Prague, Czechoslovakia (1 hr).
- R.J. Mapletoft, 1988. Embryo Transfer in Latin American Countries. CAAB Annual Meeting, Banff (1 hr).
- R.J. Mapletoft, 1987. Embryo Transfer: Your Key to Genetic Improvement. Alberta Dairy Congress, Leduc (2 hr.).
- R.J. Mapletoft, 1987. Factors Affecting Induction of Abortion or Parturition in the Bovine Species. Cornell University (1 hr.).
- R.J. Mapletoft, 1987. The Potential of Embryo Transfer for Importation of Bovine Genetics. Cooprimsem (AI organization). Osorno, Chile.
- R.J. Mapletoft, 1987. Potential of the Bovine Embryo to Prevent Spread of Infectious Diseases. Institute for Animal Reproduction, Austral University, Valdivia, Chile.
- R.J. Mapletoft, 1987. Embryo Freezing: Basic Aspects. University of Chile Medical School, Conte Hospital, Santiago, Chile.
- R.J. Mapletoft, 1987. Embryo Transfer Management. 13th Annual Food Animals Medical Conference. Ohio State University, Columbus, Ohio (1 hr.).
- R.J. Mapletoft, 1987. Superovulation in the Cow. XI Congress on Animal Reproduction. CIAVT Venado Tuerto, Argentina (1 hr.).
- R.J. Mapletoft, 1987. Synchronization of Donor Cows and Recipients. XI Congress on Animal Reproduction. CIAVT Venado Tuerto, Argentina (1 hr.).
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- R.J. Mapletoft, 1987. Disease Control and the International Movement of Embryos. IETS Meeting, Chairman of Session, Dublin.
- R.J. Mapletoft, 1987. Bovine Superovulation. IETS Meeting, Chairman of Panel Discussion, Dublin.
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- R.J. Mapletoft, 1986. Management of Pregnancy Termination in Parturient Cows and Feedlot Heifers. Herd Health Update, Coopers Agropharm, University of Guelph, Guelph, Ontario (1 hr.).
- R.J. Mapletoft, 1986. Bovine Superovulation. IETS Meeting, Panel Discussion.
- R.J. Mapletoft, 1985. Bovine Embryo Transfer. OIE Headquarters, Paris, France. (1 hr)
- R.J. Mapletoft, 1985. Bovine Embryo Transfer. World Angus Forum, Edmonton, Alberta. (1 hr)

Personnel

Jane Moker - Technician - 1985 - 1988
- 75% of time

Marilyn Braithwaite - Technician
- part time

Andrea Schumann - Technician
- 1988-1989
- 75% time

Andrzej Palasz - Professional Assoc. - 1986
- 50% of time

Vlad Pawlyshyn - Professional - casual

Curt Hagele - Professional - casual

Marshall Patterson - Professional - casual

Bryce Fleming - summer student - 1986

Dian Patt - summer student - 1987

Noeline Willmott - summer student - 1988-89

Jennifer Saunders - summer student - 1988-89

H. Wang - graduate student - M.Sc. - 1988

M. Wu - graduate student - M.Sc. - 1988

K. Xu - graduate student - M.Sc. - 1988

A. Garcia - graduate student - M.Vet.Sci. - 1989

G. Bo - graduate student

R. Mapletoft - Professor - 20%

H. Townsend - Professor - 1%

B.D. Murphy - Professor - 1%

T. Carruthers - Assistant Professor - 1%